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Novel Variant of Thyroglobulin Promoter Triggers Thyroid Autoimmunity through an Epigenetic Interferon α-modulated Mechanism*[S]

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Autoimmune thyroid diseases (AITD) arise from complex interactions between genetic, epigenetic, and environmental factors. Whole genome linkage scans and association studies have established thyroglobulin (TG) as a major AITD susceptibility gene. However, the causative TG variants and the pathogenic mechanisms are unknown. Here, we describe a genetic/epigenetic mechanism by which a newly identified TG promoter single-nucleotide polymorphism (SNP) variant predisposes to AITD. Sequencing analyses followed by case control and family-based association studies identified an SNP (−1623A→G) that was associated with AITD in the Caucasian population ($p = 0.006$). We show that the nucleotide substitution introduced by SNP (−1623A/G) modified a binding site for interferon regulatory factor-1 (IRF-1), a major interferon-induced transcription factor. Using chromatin immunoprecipitation, we demonstrated that IRF-1 binds to the 5′ TG promoter motif, and the transcription factor binding correlates with active chromatin structure and is marked by enrichment of mono-methylated Lys-4 residue of histone H3, a signature of active transcriptional enhancers. Using reporter mutations and siRNA approaches, we demonstrate that the disease-associated allele (G) conferred increased TG promoter activity through IRF-1 binding. Finally, treatment of thyroid cells with interferon α, a known trigger of AITD, increased TG promoter activity only when it interacted with the disease-associated variant through IRF-1 binding. These results reveal a new mechanism of interaction between environmental (IFNα) and genetic (TG) factors to trigger AITD.

Autoimmune thyroid diseases (AITD),[4] including Graves disease (GD) and Hashimoto thyroiditis (HT), are characterized by infiltration of the thyroid by T and B cells that react with local antigens leading to immune destruction of the thyroid in HT and production of thyroid-stimulating hormone receptor (TSHR) antibodies in GD. These result in the clinical manifestations of hypothyroidism in HT and hyperthyroidism in GD (1, 2). There is solid evidence that interactions between susceptibility genes and environmental triggers activate the sequence of cellular and humoral immune responses to thyroid antigens that cause AITD (1, 3, 4). Several environmental factors, including exposure to excess iodine, selenium deficiency, various infectious diseases, certain drugs, and pollutants have been associated with AITD (5, 6). Among these factors, interferon α (INFα), a therapeutic agent widely used for the treatment of chronic hepatitis C infection, has recently emerged as a major factor that triggers AITD (7, 8).

To date, several gene loci have been associated with AITD, including immune genes (*HLA-DR*, *CTLA-4*, *CD40*, *FOXP3*, and *CD25*) and thyroid-specific genes (TSHR and TG). Whole genome linkage screens, performed by our group (9) and others (10), have shown that the thyroglobulin (TG) locus on chromosome 8q24 is strongly linked with AITD. Moreover, TG has emerged as the only thyroid-specific gene that confers susceptibility to both GD and HT (11). Following these findings, association studies have identified several polymorphisms in the TG locus that were associated with AITD in various ethnic populations (11–15). Moreover, we performed a comprehensive sequence analysis of the entire TG coding region (48 exons) and identified a single-nucleotide polymorphism (SNP) cluster in exons 10–12 as well as an SNP in exon 33 that were significantly associated with AITD in North American Caucasians (11). Three of these four TG SNPs were nonsynonymous and resulted in amino acid substitutions in TG. However, so far the causative TG variants, and the mechanisms by which they exert their effects on gene function and contribute to the etiology of AITD, are still not known. In fact, it is possible that several TG variants are causative either singly in different patient subsets or in combination. In this study, we identified and analyzed a

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1.

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[4] The abbreviations used are: AITD, autoimmune thyroid disease; TG, thyroglobulin; GD, Graves disease; HT, Hashimoto thyroiditis; TSHR, thyroid-stimulating hormone receptor; SNP, single-nucleotide polymorphism; Q-PCR, quantitative PCR; nt, nucleotide; TF, transcription factor; TDT, transmission disequilibrium test; TI, total input; TF, transcription factor; TSS, transcriptional start site.

new variant associated with AITD that defines a *cis*-regulatory element within the TG promoter and controls gene expression. We demonstrate that this variant confers susceptibility to disease through an epigenetic interaction with IFNα.

EXPERIMENTAL PROCEDURES

Genetic Studies

Patients and Controls

The project was approved by the Mount Sinai School of Medicine Institutional Review Board. We studied 271 Caucasian AITD patients, 201 with GD and 70 with HT. 165 age- and sex-matched healthy Caucasian individuals were used as controls in the association studies. Diagnosis of GD was based on the following: (i) documented clinical and biochemical hyperthyroidism requiring treatment with or without goiter; and (ii) presence of TSHR Abs and/or diffuse thyroid scan. HT was diagnosed by the presence of clinical and biochemical hypothyroidism requiring thyroid hormone replacement with or without goiter and by the presence of anti-thyroid peroxidase Abs, with or without anti-TG Abs. Control individuals had no personal or family history of thyroid disease, had normal thyroid functions, and were negative for thyroid autoantibodies.

AITD Families

For the transmission disequilibrium test (TDT) we studied 102 families (540 individuals; for a full description of the families see Ref. 9). All families enrolled in the study were multiplex for AITD (more than one affected) and/or multigenerational. Families were ascertained through a patient with AITD, who confirmed having at least one other first degree relative with AITD. On average, our families had 5.3 members.

Sequencing Analysis

We sequenced the ~2.5-kb sequence of the TG promoter and 5′ region (16) as described previously (11). Genomic DNA was amplified using primers described in supplemental Table S1. The identified SNPs were analyzed either by a fluorescent-based restriction fragment length polymorphism method (11) or by real time PCR (Taqman ALlelic discrimination assay, Applied Biosystems) or by direct sequencing.

Case Control Association Analyses

Case control association analyses were performed by using the χ^2 and Fisher's exact tests with Yates correction. The odds ratio was calculated by the method of Woolf (17). A p value of <0.05 was considered significant.

Family-based Association Analyses

Family-based association analyses were performed using TDT. The TDT analysis was performed using Genehunter version 2.0 software (18). The TDT compares the rate of transmission of parental alleles to affected offspring with the rate expected if there is no preferential transmission (19).

Molecular Studies

Cell Lines

Human thyroid ML-1 cells were grown in DMEM + GlutaMAX-I medium (Invitrogen) supplemented with 10% FBS (GemCell). Human thyroid KTC-1 cells were grown in RPMI 1640 medium (Cellgro) supplemented with 10% FBS (GemCell) and 1% nonessential amino acid solution. Rat thyroid PCCL3 cells were grown in Nutrient Mixture F-12 Coon's Modification Medium (Sigma) supplemented with 5% FBS (GemCell) and 100 mIU TSH (Sigma), 100 μg/ml insulin (Sigma), 500 μg/ml apotransferrin (Sigma), and 100 nM hydrocortisone (Sigma). All cell lines were grown at 37 °C in 5% CO_2.

Electrophoretic Mobility Shift Assay (EMSA)

Gel Shifts with Different Cell Lines—Gel shift experiments were done using the LightShift chemiluminescent kit (Pierce) following the manufacturer's instructions. In brief, 10 fmol of double-stranded 3′-biotinylated oligonucleotide (5′-TGTACTTAAAGGAAATAAATA-3′) (G allele) was incubated with nuclear extracts from cell lines of human placenta (BeWo), human brain (A172), human liver (hepG2), rat thyroid (PCCL3), and human thyroid tissue. Briefly, binding reactions included the oligonucleotide and the nuclear extracts/nuclei, in addition to the binding buffer, glycerol, 100 mM $MgCl_2$, 1 μg/μl poly(dI·dC), 1% Nonidet P-40, as supplied in the kit. Reactions were incubated for 30 min at room temperature, and samples were then loaded on a 10% acrylamide gel. The binding reactions were then transferred to a nylon membrane (Pierce). After transfer, cross-linking was performed by placing the nylon membrane with a hand-held UV lamp equipped with a 254 nm bulb, for 15 min. The membrane was blocked overnight with the blocking buffer supplied in the kit. After overnight blocking, streptavidin/horseradish peroxidase conjugate was applied, followed by washing with the Wash Buffer. The blot was then incubated with Substrate Equilibration Buffer and developed with Substrate Working Solution that reacted with HRP. The blot was then exposed to x-ray film for detection of signal. Competition was performed by adding 2 pmol (200-fold molar excess) of double-stranded unlabeled oligonucleotide, together with 10 fmol of double-stranded 3′-biotinylated oligonucleotide, incubated with the nuclear extracts made from cell lines of human placenta (BeWo), human brain (A172), and human liver (HepG2). Binding reactions and developing conditions were the same as described above.

Competition of Unlabeled G and A Alleles with Labeled G Allele—Competition was performed by adding 200 fmol (20-fold molar excess), 500 fmol (50-fold molar excess), 1 pmol (100-fold molar excess), and 2 pmol (200-fold molar excess) of double-stranded unlabeled oligonucleotides ((5′-TGTACTTAAAGGAAATAAATA-3′) (rs180195 G allele) and 5′-TGTACTTAAAAGAAATAAATA-3′) (A allele)) together with 10 fmol of double-stranded 3′-biotinylated oligonucleotides 5′-TGTACTTAAAGGAAATAAATA-3′ (G allele) and incubating with human thyroid nuclei. Binding reactions and developing were the same as described.

Chromatin Immunoprecipitation

For nuclei preparation from ML-1 and KTC-1 cells, 6×10^6 subconfluent cells were fixed with 1% formaldehyde for 10 min at 37 °C. After fixation, cells were washed twice in ice-cold PBS (with protease inhibitors) and harvested by centrifugation at 4 °C. The nuclei were resuspended in 200 μl of nuclei lysis

buffer (Millipore) and sonicated with 20 strokes of 10-s pulses at 5% of maximum output strength on a Sonicator Ultrasonic Processor (Qsonica, LLC). ChIP was performed using a Magna ChIP assay kit (Millipore). The Abs used were as follows: anti-IRF-1 (Abcam), anti-acetyl H3 (Millipore), anti-trimethyl H3 (K4) (Millipore), and anti-monomethyl H3 (K4) (Abcam). DNA from Ab-bound chromatin, IgG control samples, and total input DNA was amplified by regular PCR using GoTaq DNA polymerase (Promega) and by quantitative PCR (Q-PCR). Q-PCR was performed in an ABI 7300 real time PCR system (Applied Biosystems Inc.) and SYBR Green was used as fluorescent dye with each sample loaded in triplicate. A comparative *Ct* method ($\Delta\Delta Ct$) was used to calculate the relative fold-change of the immunoprecipitated DNA in all samples. To estimate the IRF-1 binding to the TG promoter, Q-PCR of the immunoprecipitated chromatin was performed for a DNA sequence, including the TG rs180195, the *HLA-B* promoter, and the TSHR promoter. For estimation of the IRF-1 binding, the *Ct* values of the TG and *HLA-B* were normalized to the *Ct* values of the TSHR and expressed as a relative enrichment-fold to the TI samples. For quantitation of the DNA immunoprecipitated with anti-H3Ac, anti-H3K4me3, and anti-H3K4me1, Q-PCR was performed for a sequence encompassing the TG rs180195 and the TG proximal promoter. Relative fold-enrichment was calculated from the *Ct* values of the precipitated chromatin samples relative to the TI samples. Primers used for all ChIP PCRs are shown in supplemental Table S1.

Luciferase Reporter Constructs

To generate the pGL4.10-TG(A) vector, 2.5 kb of the human TG promoter was amplified from the phTgG8 vector containing 7.8 kb of the 5′-UTR human TG (20) and cloned into the KpnI/XhoI sites of the pGL4.10 vector (Promega). The cloned TG sequence extended from nt −2,500 to nt + 40 relative to TSS. This initial TG promoter sequence was homozygous for the A allele at position −1623 (rs18095). The G allele and an 8-bp deletion flanking this site were introduced using QuikChange XL site-directed mutagenesis kit (Stratagene) to generate the pGL4.10-TG(G) and pGL4.10-TG(del) vectors, respectively.

To generate pGL4.10-enh-TG vectors, TG proximal promoter (321 bp) was amplified from pGL4.10-TG(A) vector and cloned into the KpnI/XhoI restriction sites of the pGL4.10 vector. In addition, a 357-bp sequence encompassing the rs180195 polymorphism with A or G alleles and 8-bp deletion was amplified from the pGL4.10-TG(A), pGL4.10-TG(G), and pGL4.10-TG(del), respectively. All three fragments were cloned into the SalI/BamHI restriction sites of the pGL4.10 vector containing the TG proximal promoter. The cloned TG proximal promoter contained 321 bp and extended from nt −281 to nt +40 relative to TSS. The cloned DNA sequence, containing the rs18195 site, extended from nt −1403 to nt −1760, including the rs18195 polymorphism. Primers used to generate all pGL4.10 luciferase constructs are shown in supplemental Table S1. All constructs were verified by sequencing.

Transient transfections were conducted in human ML-1 and rat PCCL3 thyroid cell lines using Lipofectamine 2000 transfection reagent (Invitrogen). Cells were co-transfected with a mix of 200 ng of firefly pGL4.10 luciferase reporter vectors and 4 ng of *Renilla* luciferase reporter pGL4.74[*hRluc*/TK] (Promega). 24 or 48 h after transfection, we harvested the cells and measured the luciferase activity using the Dual-Luciferase™ reporter assay system (Promega). Firefly luciferase values were divided by *Renilla* luciferase values for each transfection, and data from at least three transfections were averaged. Statistical differences between vectors harboring the two alleles were determined using an independent *t* test.

siRNA-mediated Inhibition of IRF-1

ML-1 cells were co-transfected with pGL4.10-TG vectors harboring either A or G alleles, pGL4.74[*hRluc*/TK] control plasmid, and siRNA against IRF-1 (Ambion) to a final concentration of 100 nM using Lipofectamine 2000 transfection reagent. For a positive control, we used co-transfection of the pGL4.10 luciferase vectors and *Renilla* control vector with siRNA against Nkx2.1. We harvested the cells 48 h after transfection and measured the luciferase activity using the Dual-Luciferase™ reporter assay system. Statistical differences between cells transfected with siRNA and untransfected cells were determined using an independent *t* test.

IFNα Activation Assays

ML-1 cells were cultured in 6-well plates (5×10^5 cells/well) 24 h before adding IFNα (Millipore) to a final concentration of 1×10^3, 5×10^3, and 1×10^4 units/ml. ML-1 cells were cultured in the presence of IFNα for an additional 24h and 48 h. Cells were harvested, and RNA was extracted using TRIzol (Invitrogen), treated with RQ1 RNase-Free DNase (Promega), and reverse-transcribed using Superscript First-Strand Synthesis System (Invitrogen). RNA expression was measured by QRT-PCR using the TaqMan Gene Expression Assay (Applied BioSystems) for *IRF-1* (Hs00971965-m1), TG (Hs0079359-m1), and *GAPDH* (Hs99999905-m1). Relative quantification of gene expression was assessed by real time QRT-PCR carried out in a PRISM 7200 sequence detection system. *GAPDH* was used as an endogenous control, and all results were analyzed using a comparative **Ct** method. Results from three independent transfections were used to calculate statistical differences between cells treated with and untreated with IFNα using an independent *t* test.

For analysis of the IFNα effect on TG promoter activity, pGL4.10-TG(A) and pGL4.10-TG(G) vectors were transfected into ML-1 as described above. Transfected cells were treated with 1×10^3 units/ml IFNα for additional 24 h. Promoter activity was expressed as a ratio of firefly to *Renilla* luciferase values. Statistical differences between vectors harboring the two alleles were determined using an independent *t* test.

RESULTS

Association of an A/G SNP in the 5′-Untranslated Region of the TG Gene with AITD—The human TG promoter was described in 1985 (16) and characterized by several groups (21–23). The TG proximal promoter extends ~200 bp upstream from the start codon (Fig. 1*a*) and includes the binding sites for two thyroid-specific TF, TTF1, and Pax8 (23). However, regulatory elements such as a cAMP-responsive element enhancer

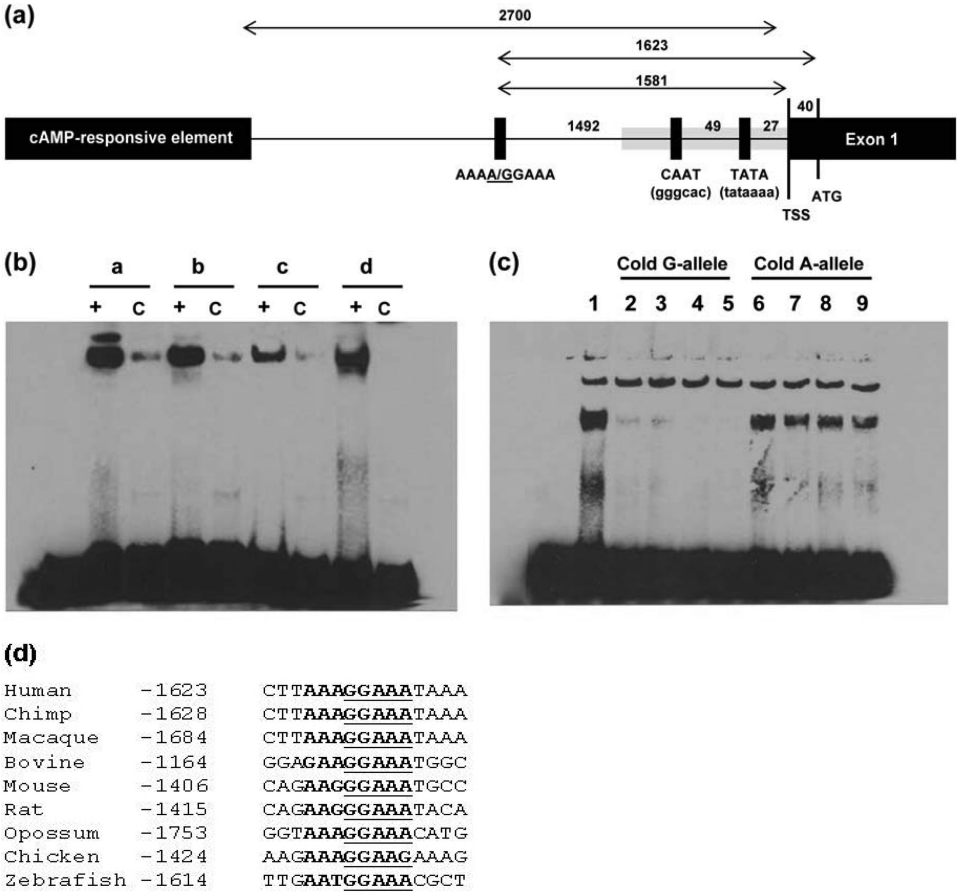


FIGURE 1. **rs180195 SNP is located within a transcription factor-binding site.** *a,* structure of the promoter and 5′ upstream region of the human TG gene. The *gray box* indicates the proximal promoter, extending ~170 bp upstream of the TSS and comprising a TATA-box and a CAAT-box. There is a documented cAMP-responsive element 2.7 kb upstream of the TSS (22). The SNP examined in this paper is located 1,623 bp upstream ATG or 1,581 bp upstream of the TSS. *b,* electromobility shift assays (*EMSA*) using oligonucleotides flanking the rs180195 G variant. *c,* G allele with nuclear extract and cold competitor at 200 fold excess; +, G allele with nuclear extract; *lanes a,* human placenta (BeWo) cell line; *lanes b,* human brain (A172) cell line; *lanes c,* human liver (hepG2) cell line; *lanes d,* rat thyroid (PCCL3) cell line. Nuclear extracts from each cell line were incubated with 20 pmol of dsDNA. *c,* EMSA competition experiment demonstrating specific binding of a nuclear factor to the rs180195 G allele. Nuclear extracts from human thyroid tissue were incubated with 20 pmol of labeled dsDNA and different concentrations of competitor. *Lane 1,* rs185190 G allele without competitor; *lanes 2–5,* rs185190 G allele with increasing amounts of rs185190 G allele competitor (*lane 2,* 10-fold molar excess; *lane 3,* 20-fold molar excess; *lane 4,* 50-fold molar excess; *lane 5,* 100-fold molar excess); *lanes 6–9,* rs185190-labeled G allele with increasing excess of rs185190 A allele competitor (*lane 6,* 10-fold molar excess; *lane 7,* 20-fold molar excess; *lane 8,* 50-fold molar excess; *lane 9,* 100-fold molar excess). Only the rs185190 G allele competed off the labeled G allele. *d,* conservation of the disease-associated G polymorphism rs180195 in vertebrates. Sequences from vertebrates that are homologous to the TG motif region were selected using BLAST.

have been located as far as ~3 kb from the TSS (Fig. 1*a*) (22). We sequenced the 2.5-kb segment upstream of the TG start codon in 30 individuals (20 AITD patients and 10 healthy controls). This region includes the 5′-UTR, the promoter, and other regulatory regions of the TG gene (Fig. 1*a*). We identified three new SNPs as follows: 1) a C/T transition in position −1984 (rs180193); 2) an A/G transition in position −1714; and 3) an A/G transition in position −1623 (rs180195) (note that two of these SNPs were later identified by the HapMap consortium and assigned rs numbers; therefore, from here on we use their universal rs numbers). All SNP positions are relative to the start codon. We tested allele and genotype frequencies for all three SNPs in 271 Caucasian AITD patients (201 with GD and 70 with HT) and 165 population-matched controls. SNPs at positions −1714 and −1984 showed no difference in allele or genotype frequencies between AITD patients and controls (Table 1). However, −1623 A/G SNP (rs180195) showed a significant increase in the frequency of the G allele ($p = 0.006$) and the G/G genotype ($p = 0.03$, odds ratio = 1.6) in AITD patients compared with controls (Table 1).

To confirm the association of rs180195 with AITD, we performed a family-based association study using a TDT in a cohort of 102 multiplex families (9). TDT analysis confirmed the association of rs180195 with AITD demonstrating a significant excess transmission of the G allele to AITD patients ($p = 0.034$, Table 1). Interestingly, when we included only AITD patients with a young age of onset (age of onset ≤30) the association became notably stronger ($p = 2.0 \times 10^{-3}$).

rs180195 Site Marks a 5′-Regulatory Element in TG Promoter—To investigate whether rs180195 was located within a regulatory element containing a TF-binding site, we first performed EMSA on oligonucleotides designed to include only the disease-associated allele (G allele). We generated nuclear extracts from human thyroid tissue, and from the following cell lines: rat thyroid (PCCL3), human placenta (BeWo), human glial (A172), and human hepatocyte

TABLE 1
Association of *TG* promoter SNPs with autoimmune thyroid disease

SNP position in Tg promoter	Allele	AITD Patients No. of Chromosomes (%)	Controls No. of Chromosomes (%)	*P*-value	Odds ratio	Genotype	AITD Patients No. of Individuals (%)	Controls No. of Individuals (%)	*P*-value	Odds ratio
-1623	G	325 (60.0)	156 (50.3)	0.006	1.5	GG	97 (35.8)	40 (25.8)		
	A	217 (40.0)	154 (49.7)			AG	131 (48.3)	76 (49.0)	0.03[a]	1.6[a]
						AA	43 (15.9)	39 (25.2)		
-1714	G	461 (88.0)	145 (89.5)	NS[d]	NS	GG	204 (77.9)	66 (81.5)		
	A	63 (12.0)	17 (10.5)			AG	53 (20.2)	13 (16.0)	NS[b]	NS[b]
						AA	5 (1.9)	2 (2.5)		
-1984	C	310 (57.8)	168 (50.9)	0.047	1.3	CC	87 (32.5)	42 (25.5)		
	T	226 (42.2)	162 (49.1)			CT	136 (50.7)	84 (50.9)	NS[c]	NS[c]
						TT	45 (16.8)	39 (23.6)		

TDT Analysis of SNP -1623	Allele	Transmitted	Untransmitted	*P*-value
All AITD patients	G	45	27	0.034
	A	27	45	
Patients with age of onset ≤ 30 years	G	26	8	$2.0X10^{-3}$
	A	8	26	

[a] *p* value and odds ratio are for GG *versus* AA + AG genotypes.
[b] *p* value and odds ratio are for GG + AG *versus* AA genotypes.
[c] *p* value and odds ratio are for CC + CT *versus* TT genotypes.
[d] NS means not significant.

TABLE 2
rs180195 genotype in human thyroid cell lines

Cell line name	Tumor tissue origin	rs180195 genotype
KTC1	Papillary	A/G
TPC1	Papillary	G/G
K1	Papillary	G/G
BCPAP	Papillary	G/G
FTC133	Follicular	G/G
ML1	Follicular	G/G
SW1736	Anaplastic	G/G
8505C	Anaplastic	G/G
BHT101	Anaplastic	A/G
hTh74	Undifferentiated	A/G

(HepG2) cells. For all tested extracts, we found that a nuclear transcription factor binds specifically to the 21-bp oligonucleotides matching the rs180195 G allele-TGTACTTAAA**G**GAAATAAATA (Fig. 1*b*). This suggested that a ubiquitously expressed transcription factor targets the TG promoter motif (Fig. 1*b*). The possibility that the presence of the G or A allele at the rs180195 site affected the binding of nuclear proteins was further analyzed by EMSA using specific competitors for the labeled G allele. The "cold" rs180195 G allele competed off the labeled G allele. In contrast, the cold rs180195 A allele (TGTACTTAAA-**A**GAAATAAATA) did not compete off the labeled G probe demonstrating a specific protein-DNA interaction for the rs180195 G allele (Fig. 1*c*).

We then used the JASPAR motif finder program (24) to search for potential TF-binding motifs that match the TG promoter 5-nucleotide sequence present at the rs180195 site, **G**GAAA or **A**GAAA. Potential binding motifs for two TFs were identified, ETS1 (% score of 74.98 for a threshold of 80%) and IRF-1 (% score of 57.12 for a threshold of 80%). The core sequences (GGAAA and AGAAA) around the rs180195 site were also tested for conservation in vertebrates by homology searches using BLAST (25) against genome sequences, and manually aligning the syntenic regions around the motif. Analysis of the GGAAA sequence at the 5′ of the TG gene demonstrated a remarkable conservation among vertebrates (Fig. 1*d*). Interestingly, we found that only the disease-associated allele (G) is conserved in vertebrates, whereas the A allele (disease-protective) is unique to humans, suggesting that the A allele may be a recent adaptive adjustment to the disease. To confirm the conservative nature of the rs180195 site motif, we sequenced the region in 23 mouse strains and found complete conservation of the GGAAA sequence with no evidence of polymorphisms in any strain.

rs180195 Disease-associated G Allele Binds Transcription Factor IRF-1 in Vivo—To discriminate between the two possible TF candidates identified by bioinformatic analysis, we performed ChIP in the human thyroid cell line, ML-1 (26), using antibodies for ETS1 and IRF-1. We selected the ML-1 human thyroid cell line because sequencing of genomic DNA from ML-1 cells showed the presence of the GG genotype at rs180195 (Table 2), and the cell line also showed robust expression for both TG and *IRF-1* genes (Fig. 2). ChIP analyses demonstrated that the TF IRF-1 bound to the TG promoter motif at the rs180195 site (Fig. 3*a*). However, no binding of ETS-1 to the TG rs180195 site was found by ChIP (data not shown). Q-PCR of the immunoprecipitated chromatin from ML-1 cells showed

an ~14-fold enrichment of IRF-1 antibody at the 5′ TG site compared with an irrelevant DNA sequence from the TSHR promoter (Fig. 3*b*). To further investigate the association between IRF-1 binding and rs180195 G allele, we screened nine additional human thyroid cell lines (27) by DNA sequencing (Table 2). For the ChIP assays, we chose KTC-1, a human papillary thyroid cell line that contains the AG genotype at rs180195 and expresses mRNA for TG and *IRF-1* (Fig. 2) (Intriguingly, none of the cell lines had the AA genotype at rs180195.). Q-PCR showed an ~8-fold increase in IRF-1 binding at the rs180195 site compared with an irrelevant DNA fragment from TSHR promoter (Fig. 3*c*). Quantitative assessment of the IRF-1 binding in ML-1 and KTC-1 cells showed ~2-fold increased antibody enrichment with the GG allele (ML-1) compared with the AG allele (KTC-1) indicating an allele-specific binding of IRF-1. Because none of the established thyroid cell lines (27) we tested harbored the AA genotype at rs180195 (Table 2), we could not test *in vivo* binding of IRF-1 to the TG promoter when the AA genotype was present at rs180195 site. Together, these results showed preferential binding of IRF-1 to the TG promoter containing the G allele and suggested differential regulation of TG transcription by the two alleles.

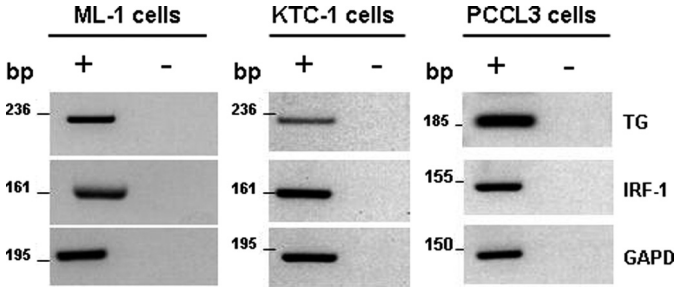


FIGURE 2. **mRNA expression of** TG **and *IRF-1* in ML-1 and KTC-1 human thyroid cell lines and PCCL3 rat thyroid cell line.** mRNA expression of TG and *IRF-1* was assessed by RT-PCR. GAPDH was used as internal control of gene expression. Both TG and *IRF-1* genes were expressed in all tested cell lines.

rs180195 Disease-associated G Allele Confers Increased TG Promoter Activity—To differentiate between the effects of the two rs180195 alleles (A or G) on the activity of the TG promoter, we tested both the A and the G promoter alleles of TG by reporter mutations and luciferase assays. We cloned the 2.5-kb human TG promoter and 5′ upstream region containing the rs180195 A allele into the pGL4.10 luciferase vector (Fig. 4*a*). The rs180195 G allele and an 8-bp deletion flanking this site

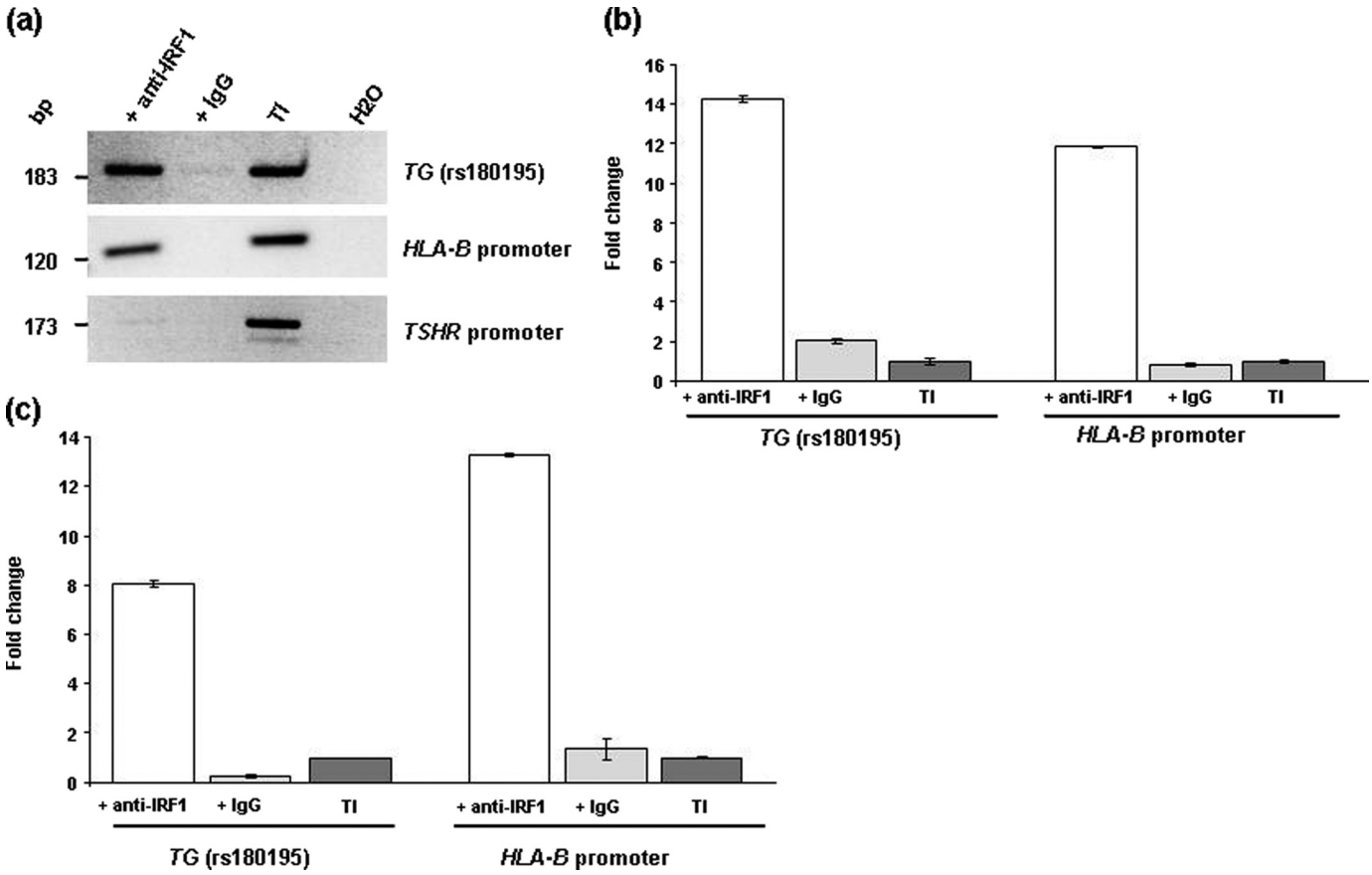


FIGURE 3. **Functional IRF-1-binding element is present in the** TG **promoter at rs180195 site.** *a,* IRF-1 binds to the endogenous TG promoter. ChIP was performed with anti-IRF-1 Abs using ML-1 human thyroid cells. PCR of the immunoprecipitated chromatin was positive for the sequence encompassing rs180195 site in the TG promoter. Positive control included PCR of a known IRF-1 site within the *HLA-B* promoter. Negative control included amplification of an irrelevant sequence from the TSHR promoter. ChIP controls included chromatin immunoprecipitated with IgG (+*IgG*) and total input DNA (*TI*). *b,* quantitation of IRF-1 binding to the TG promoter in ML-1 thyroid cells homozygous for the rs180195 G allele. Estimation of IRF-1 binding was obtained from the *Ct* values of TG and *HLA-B* normalized to the *Ct* values of TSHR and expressed as a relative enrichment-fold to the TI samples. Results are presented relative to TSHR. *c,* quantitation of IRF-1 binding to the TG promoter in KTC-1 cells heterozygous for the rs180195 A/G variant. Evaluation of IRF-1 binding was done as in *b*. Enrichment of anti-IRF-1 Abs relative to total input at the TG rs180195 site was decreased by 2-fold in KTC-1 cells compared with ML-1 cells, suggesting that IRF-1 binds specifically to the G allele.

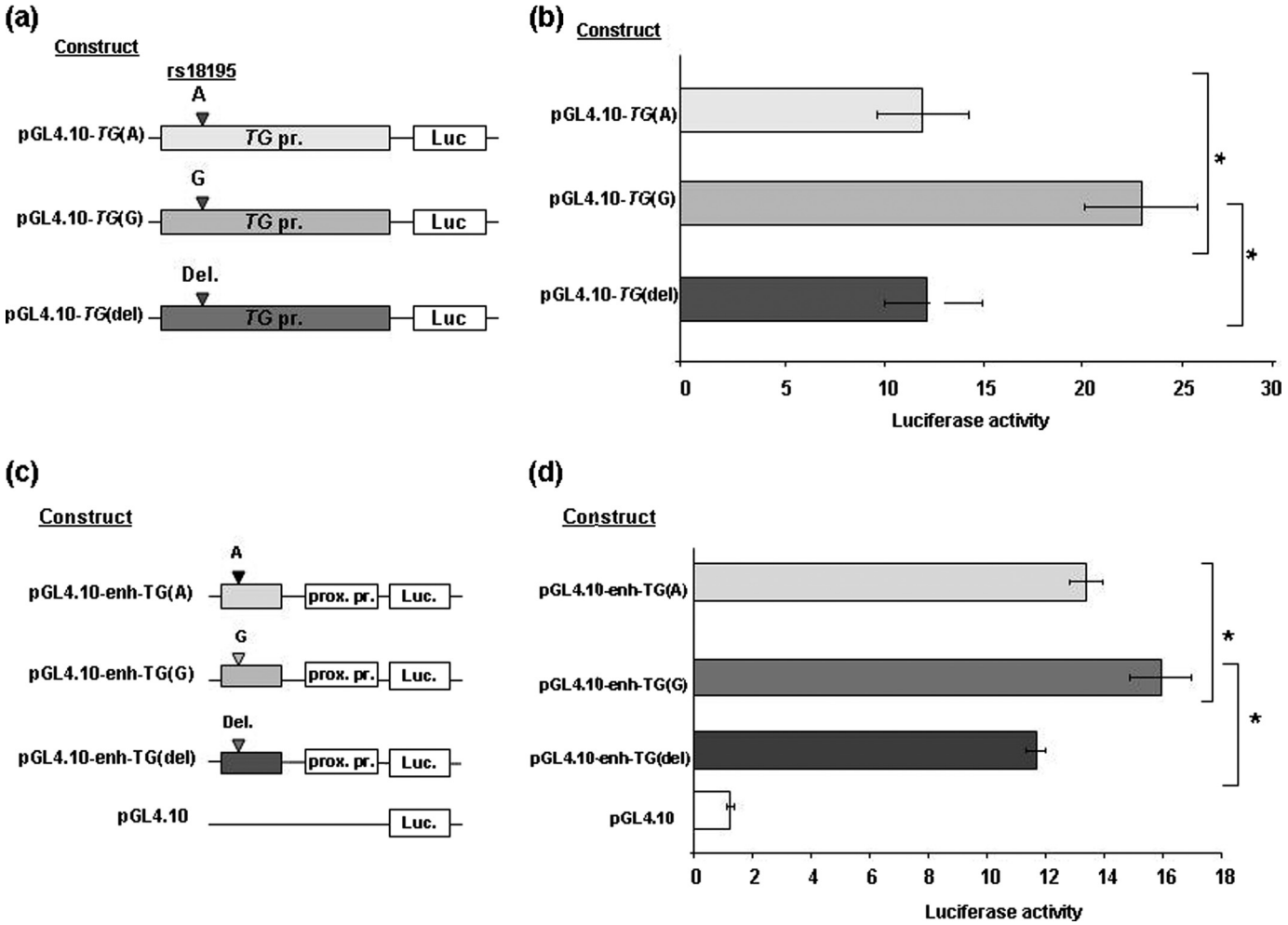


FIGURE 4. **Functional analysis of IRF-1-dependent** TG **promoter activity.** *a*, constructs. 2.5-kb sequence, including the rs180195 A allele at the 5′ upstream TG gene, was cloned into pGL4.10 vector to generate pGL4.10-TG(A). The rs180195 G allele as well as an 8-bp deletion flanking the disease-associated polymorphism were introduced by site-directed mutagenesis to generate pGL4.10-TG(G) and pGL4.10-TG(del), respectively. *b*, presence of the rs180195 G allele confers increased TG promoter activity. Each reporter construct was transfected into PCCL3 thyroid cells, and relative luciferase activities were measured. Luciferase (*Luc*) activity of pGL4.10-TG(G) harboring the disease-associated allele was increased by 2-fold compared with pGL4.10-TG(A) ($p = 0.0072$) and pGL4.10-TG(del) ($p = 0.0077$) constructs. Each experiment was performed with 12 duplicates. Data represent the mean ± S.D. of three independent experiments. *c*, constructs. TG proximal promoter (321 bp) was cloned into the promoter region of the pGL4.10 vector. A 356-bp sequence encompassing rs180195 and containing either the A allele, the G allele, or the 8-bp deletion was cloned in the enhancer region of the same vector to generate pGL4.10-enh-TG(A), pGL4.10-enh-TG(G), and pGL4.10-enh-TG(del). *d*, TG promoter activities by luciferase assay. Each reporter construct was transfected into ML-1 thyroid cells, and relative luciferase activities were measured. Luciferase activity of the pGL4.10-enh-TG(G) harboring the disease-associated allele was increased by ~1.2- and ~1.3-fold compared with pGL4.10-enh-TG(A) ($p = 0.032$) and pGL4.10-TG(del) ($p = 0.011$), respectively. Data represent the mean ± S.D. of three independent experiments. Statistical differences between various constructs were determined using a *t* test for independent samples (*, $p < 0.05$).

were created by site-directed mutagenesis, and these three vectors (Fig. 4*a*) were used to transfect PCCL-3 rat thyroid cells. PCCL3 cells showed robust mRNA expression of TG and *IRF-1* (Fig. 2). Levels of luciferase expression driven by the A allele were similar to the 8-bp deletion that eliminated the entire rs180195 site, suggesting that the A allele had no effect on the TG promoter activity (Fig. 4*b*). However, the presence of the G allele increased the luciferase activity by ~2-fold compared with the A allele ($p = 0.007$) and the 8-bp deletion ($p = 0.007$), demonstrating that the disease-associated allele conferred increased constitutive activity of the TG promoter (Fig. 4*b*). To further prove that this increased TG promoter activity was due to the presence of the rs180195 polymorphism and to exclude nonspecific effects that may occur in the 2.5-kb cloned fragment, we created three other related constructs in which the TG proximal promoter (321 bp) was cloned into the promoter region of the pGL4.10 vector, and only a short sequence (357 bp) containing the rs180195 polymorphism with A or G alleles and 8-bp deletion was cloned into the enhancer position of the luciferase vector (Fig. 4*c*). Transfection of these constructs into thyroid ML-1 cells showed that the presence of the rs180195 G allele conferred increased TG promoter activity compared with the A allele ($p = 0.03$) or with the 8-bp deletion ($p = 0.01$), exactly as we observed with the entire 2.5-kb fragment (Fig. 4*d*). These results confirmed the regulatory role of the rs180195 disease-associated G variant on TG promoter activity.

IRF-1 Regulates Activity of the Disease-associated Promoter Variant—To test if the allele-specific effect on TG promoter activity depended on IRF-1 expression, we co-transfected the TG luciferase constructs harboring the A and G alleles (Fig. 4*a*) with short interfering (si) RNAs targeting IRF-1 in ML-1 cells. Transfection of the IRF-1 siRNA significantly down-regulated

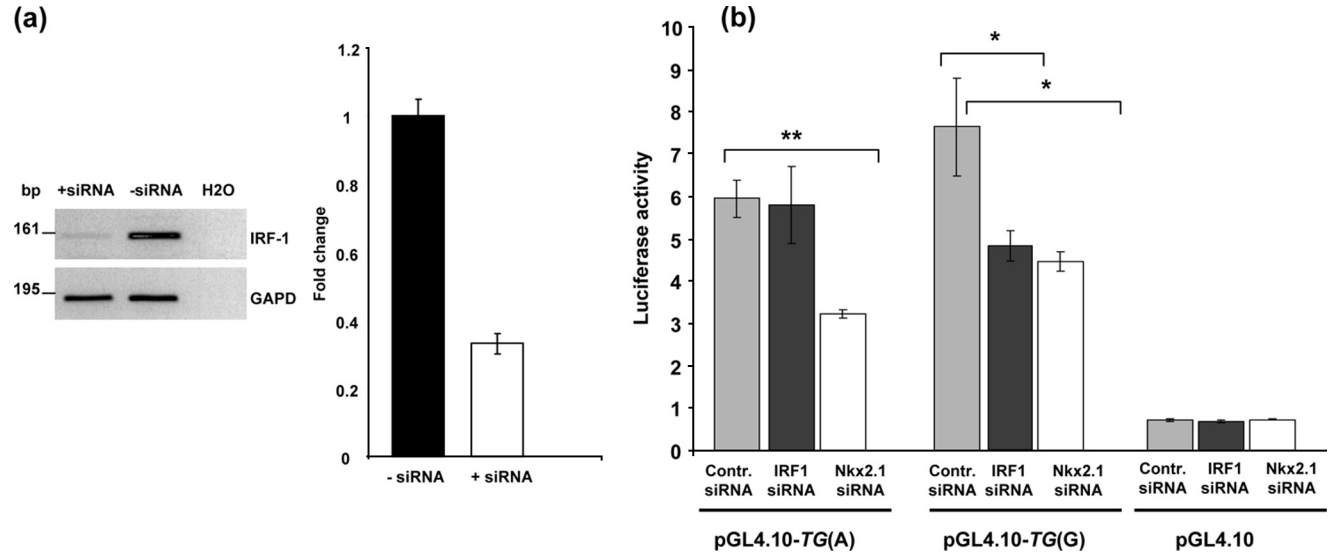


FIGURE 5. *a,* reduced IRF-1 mRNA expression in ML-1 cells treated with IRF-1 siRNA. mRNA expression of IRF-1 was assessed by regular RT-PCR and QRT-PCR. *b,* knockdown of IRF-1 by siRNA reduces the TG promoter activity only in the presence of rs180195 G allele. pGL4.10-TG(A) and pGL4.10-TG(G) constructs were co-transfected with IRF-1 siRNA into ML-1 thyroid cells, and luciferase activities were measured. IRF-1 siRNA decreased the relative luciferase activity by 1.5-fold ($p = 0.04$) only when the disease-associated allele (G) but not when the A allele was present at the rs180195 site. Controls (*Contr.*) included co-transfection of each reporter construct with siRNA against Nkx2.1 (TTF-1), a TF shown to activate TG promoter activity (23). *, < 0.05; **, < 0.005.

the *IRF-1* gene expression as evaluated by regular RT-PCR and QRT-PCR (Fig. 5*a*). TG promoter activity was not influenced by IRF-1 siRNA when the A allele was present but was decreased by 1.5-fold ($p = 0.04$) in the presence of the G allele, demonstrating that the rs180195 G allele increased TG promoter activity through the binding of IRF-1 (Fig. 5*b*).

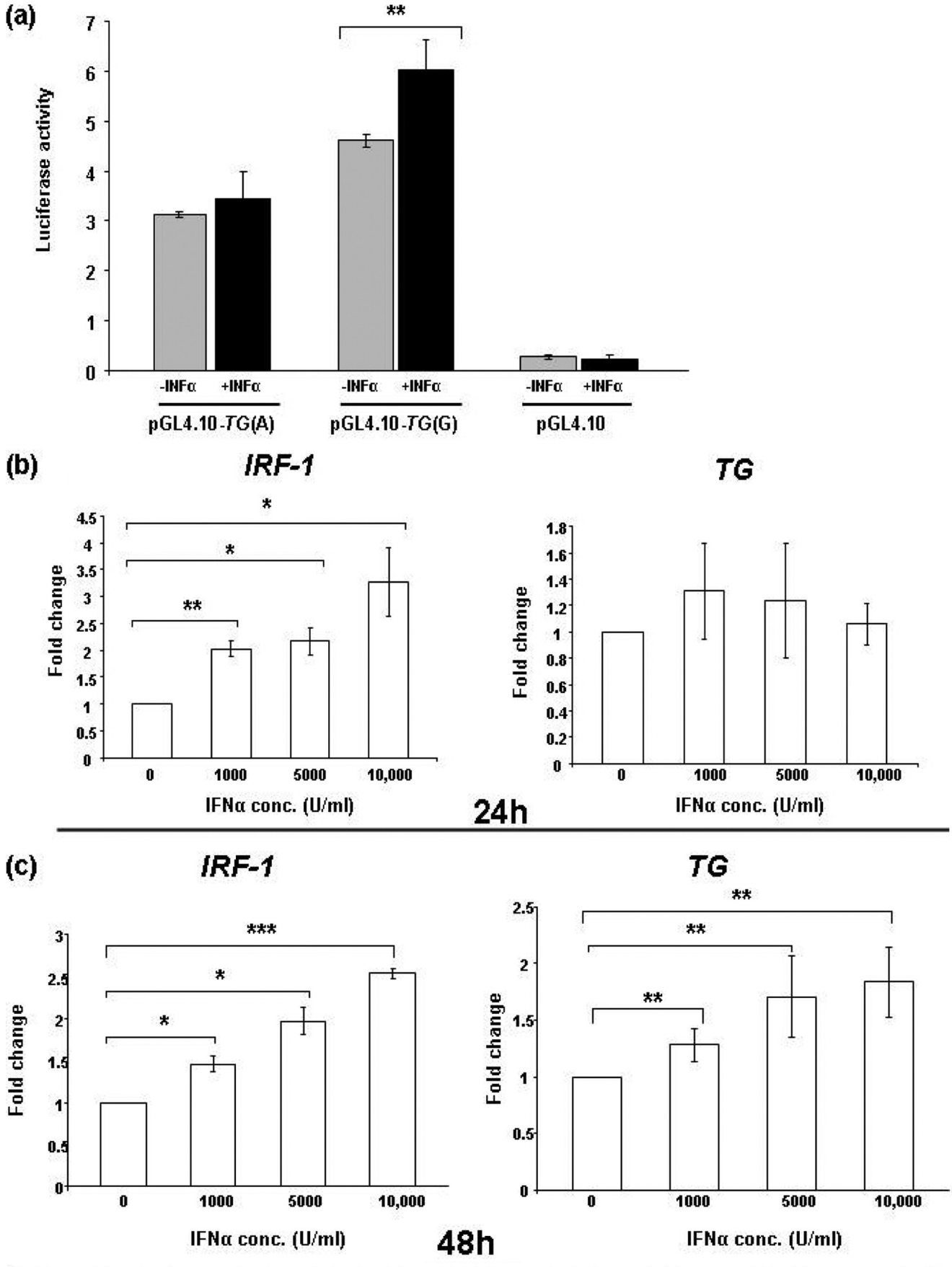
INFα Up-regulates Expression of the Disease-associated TG Variant—IRF-1 is induced by IFNα upon binding to the IFN receptor (28), and it functions as an important regulator of cellular responses to IFNα by affecting transcription of IFNα-inducible genes (29). We tested whether IFNα could activate TG transcription through IRF-1 binding and whether this activation was dependent on the rs180195 genotype. IFNα treatment of ML-1 cells, transfected with the TG promoter luciferase constructs containing the A and G alleles, up-regulated luciferase expression only when the G allele was present in the TG promoter ($p = 0.0004$) (Fig. 6*a*). To confirm the luciferase results, we tested whether IFNα can induce endogenous TG transcription in thyroid cells harboring the GG genotype through activation of IRF-1. We found that 24 h of exposure of ML-1 cells (GG at rs180195) to different concentrations of IFNα resulted, as expected, in increased mRNA expression of IRF-1 (Fig. 6*b*). Moreover, treatment of ML-1 cells with IFNα for 48 h also increased the mRNA levels of endogenous TG (Fig. 6*c*), confirming our luciferase data. Combined, these results suggested that IFNα modulates TG promoter activity via IRF-1 binding to the rs185190 motif and that this effect is dependent on the rs185198 genotype.

IRF-1 Binding Correlates with Histone Markers of Active Chromatin—To investigate if IRF-1 binding to the rs180195 site was associated with active chromatin structure, we performed ChIP assays in ML-1 cells using markers of histone modifications. ChIP analyses in ML-1 thyroid cells using anti-acetyl histone H3 antibody demonstrated that the chromatin around both the TG proximal promoter and the rs180195 site was enriched in histone H3 acetylation (H3Ac), a marker of active transcriptional elements (Fig. 7). ChIP analyses using anti-trimethyl H3 (Lys-4) and anti-monomethyl H3 (Lys-4) Abs showed that chromatin at the TG proximal promoter is characterized by enrichment of both H3K4me3 and H3K4me1 (Fig. 7). However, the chromatin at the rs180195 motif was marked by depletion of H3K4me3 and enrichment of H3K4me1 characteristic of a functional enhancer (Fig. 7).

DISCUSSION

An increasing body of evidence, including case control association studies and family-based linkage screens, demonstrate that TG is a major AITD susceptibility gene (11–15, 30–32). Although a number of TG variants have been reported to be associated with AITD, the causative variant or variants and the mechanisms by which these polymorphisms contribute to the complex etiology of AITD are not known. For a comprehensive understanding of the role of TG gene variants in the etiology of autoimmune thyroiditis, we investigated polymorphisms in noncoding regulatory regions of the human TG gene. Here, we present a new TG gene variant we found associated with AITD, and we demonstrate that the disease-associated G allele confers increased promoter activity through the binding of the TF IRF-1.

We have shown that the newly identified SNP is located within a TG 5′-regulatory element characterized by histone markers of active chromatin. Reversible modifications of amino-terminal tails of histones, particularly acetylation and methylation, play an important role in the regulation of transcription (34–37). Although multiple histone modifications can be associated with transcriptional regulatory elements, specific chromatin signatures of promoters and enhancers have been proposed. Generally, high levels of acetylation, as well as methylation of the Lys-4 residue of histone H3, are detected in the promoter regions of transcriptionally active genes (36–38).


(a)
Luciferase activity
-INFα
+INFα
pGL4.10-TG(A)
pGL4.10-TG(G)
pGL4.10
(b)
IRF-1
TG
Fold change
IFNα conc. (U/ml)
24h
(c)
IRF-1
TG
Fold change
IFNα conc. (U/ml)
48h

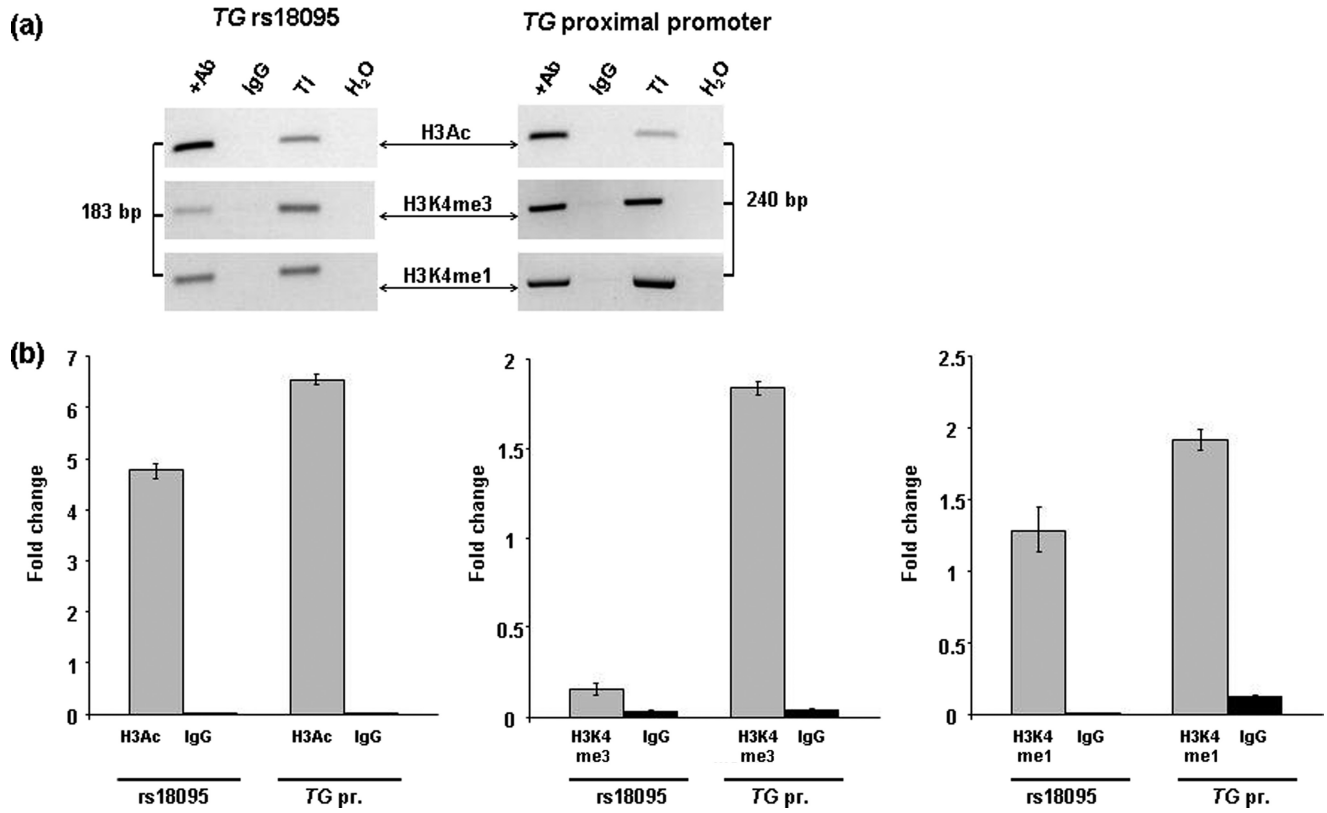


FIGURE 7. **Histone markers at the rs180195 site in ML-1 thyroid cells.** Chromatin from ML-1 cells was immunoprecipitated with anti-acetylated histone H3 (*H3Ac*), anti-trimethyl histone H3(K4) (*H3K4me3*) and anti-monomethyl histone H3(K4) (*H3K4me1*) Abs. Controls included precipitation with IgG (+*IgG*), DNA from total input (*TI*), and H_2O. *a,* PCR of DNA from chromatin immunoprecipitated with H3Ac, H3K4me3, and H3K4me1. PCR was done using primers for the rs180195 site and for the TG proximal promoter. *b,* quantitative-PCR for the rs180195 site and for the TG proximal promoter. Quantitation of enrichment for each antibody was obtained from the *Ct* values and calculated as an enrichment-fold relative to TI samples. rs180195 site (position −1623 from the start codon) was characterized by depletion of H3K4me3 and enrichment of H3K4me1 compared with the TG proximal promoter (position −224 from the start codon). TG proximal promoter showed increased trimethylation and monomethylation of histone H3 at Lys-4 residue. Chromatin regions of both the rs180195 site and the TG proximal promoter showed elevated levels of H3Ac.

More specifically, it has been shown that although trimethylation of the Lys-4 residue of histone H3 (H3K4me3) is enriched at 5′ regions of the active genes and appears to mark exclusively active promoters (35, 37), monomethylation of the Lys-4 residue of histone H3 (H3K4me1) outside the promoter regions is characteristic of functional enhancers (37, 39, 40). Because the chromatin around the rs180195 site was characterized by enhancer-specific histone modifications (enrichment of H3K4me1 and depletion of H3K4me3) and correlated with IRF-1 binding to the 5′ site, we propose that the disease-associated variant defines an active enhancer element.

Our study points to a novel mechanism of gene-environment interaction in triggering thyroid autoimmunity. INFα is a critical cytokine, produced by cells in response to viral infection, which mediates its biological effects through induction of specific genes. We demonstrated that IFNα interacts with TG through IRF-1, in a genotype-dependent manner, to modulate TG transcription. The fact that TG levels increased by 30–100% following IRF-1 induction when the G allele was present suggests that other TG regulatory elements may exist 5′ of the TG gene. The transcriptional effects we observed with IRF-1 are most likely biologically significant. However, future studies assessing the role of IRF-1 regulation of TG gene transcription in the pathogenesis of thyroiditis may reveal the biological significance of the interaction between IRF-1 and the TG promoter in differential TG expression.

TG is the key autoantigen in AITD and it accounts for ~80% of the total thyroidal protein (41). Indeed, AITD are characterized by high prevalence of anti-TG antibodies, and the classical animal model of HT, experimental autoimmune thyroiditis, is induced by immunizing mice with TG and adjuvant (42–44). Moreover, recently Chen *et al.* (45) have shown that in a spontaneous model of autoimmune thyroiditis, TG antibodies arise first and thyroid peroxidase antibodies only later, suggesting that the autoimmune response to TG is the first event in the development of thyroid autoimmunity. This notion is also sup-

FIGURE 6. **INFα stimulates expression of** TG **in the presence of the disease-associated variant.** *a,* specific effect of IFNα on pGL4.10-TG(G) construct activity. Treatment of ML-1 cells with IFNα increased the TG promoter activity when G allele was present at rs180195 site ($p = 0.0003$). *b* and *c,* effects of IFNα on endogenous *IRF-1* and TG expression in ML-1 cells. ML-1 cells were treated with three different concentrations of IFNα (1,000, 5,000, and 10,000 units/ml), and mRNA expression of *IRF-1* and TG was assessed after 24 h (*b*) and 48 h (*c*). mRNA levels were assessed by quantitative (Q)RT-PCR using a $\Delta\Delta Ct$ method and normalized to *GAPDH* mRNA levels. Results are presented as mean fold-change ± S.D. of three independent experiments; significant differences were determined using a *t* test for independent samples (*, $p \leq 0.05$; **, $p \leq 0.005$; ***, $p \leq 0.0005$). *IRF-1* mRNA levels were significantly increased at all concentrations of IFNα after 24 and 48 h. TG mRNA expression was significantly up-regulated only after 48 h of INFα treatment of ML-1 cells.

ported by our genetic studies (11). Therefore, up-regulation of TG expression, by IFNα, could propagate a TG-targeted autoimmune response in the setting of viral infections, which are known to induce IFNα secretion.

IFNα is also an essential component of the therapy for many diseases and is extensively used in the treatment of chronic hepatitis C (7, 46, 47). However, despite its success, IFNα has numerous side effects ranging from mild flu-like symptoms to life-threatening conditions that can require dose reduction or discontinuation of the drug. Among these, thyroid disease is the most common autoimmune condition associated with IFNα treatment because 5–15% of hepatitis C patients treated with IFNα develop clinical thyroiditis and 20–40% develop thyroid antibodies (7, 46, 47). It is believed that a genetic predisposition to thyroid autoimmune disease may be necessary for the development of interferon-induced thyroiditis (48). The mechanisms through which IFNα treatment triggers thyroiditis in patients with hepatitis C are not known, but recent data from our group have shown that IFNα itself can cause thyroid cell necrosis and up-regulation of cytokines, which can trigger autoimmune thyroiditis by a bystander mechanism (46, 49). Moreover, we have shown that IFNα induces expression of all thyroid-specific genes (TG, TSHR, thyroid peroxidase, and *NIS*) in thyroid cells (49). Therefore, a picture is emerging whereby conditions that increase intra-thyroidal levels of IFNα, such as viral infections, can trigger AITD by up-regulating TG expression in the setting of thyroid cell damage that can trigger an autoimmune response by bystander mechanisms (50).

In this study we present for the first time a potential mechanism by which IFNα can trigger AITD in genetically susceptible individuals. The proposed mechanism works through a genetic/epigenetic interaction with a disease-associated promoter variant. Conditions associated with increased local production of IFNα (*e.g.* viral infections) would lead to increased expression of autoantigens in susceptible individuals. If this model is confirmed, it may represent a more generalized model for epigenetic interactions between environmental triggers, such as infections, and gene variants in the induction of autoimmune disease, as has been shown in the case of Crohn disease (33).

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